AD_____

Award Number: W81XWH-08-1-0178

TITLE: Understanding and targeting cell growth networks in breast cancer

PRINCIPAL INVESTIGATOR: Jason D. Weber, Ph.D.

CONTRACTING ORGANIZATION: Washington University School of Medicine
St. Louis, MO 63110

REPORT DATE: April 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-04-2012	Annual	17 MAR 2011 - 16 MAR 2012

4. TITLE AND SUBTITLE
Understanding and targeting cell growth networks in breast cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0178

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jason D. Weber, Ph.D.

E-Mail: jweber@dom.wustl.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Washington University School of Medicine
St. Louis, MO 63110

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Abstract on next page.

15. SUBJECT TERMS
Subject terms on next page.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	20	19b. TELEPHONE NUMBER *(include area code)*

14. ABSTRACT

In this fourth annual review, we have demonstrated that translation plays a huge role in determining tumor initiation by oncogenes. We have found that the ARF tumor suppressor is translationally regulated by mTORC1 signals and that ARF in turn negatively regulates Drosha mRNA translation. This is important because loss of Drosha results in an inability to transform immortal cells with oncogenic RasV12 alleles. This implies that Drosha provides a pro-growth setting for future oncogenic events and that inhibiting Drosha function could be an important therapeutic avenue. We have also identified a second translational target of ARF: the DHX33 DEAD-box RNA helicase. Much like Drosha, ARF prevents DHX33 translation. DHX33 expression is required for efficient RasV12 translation, causing us to label ARF as a master regulator of oncogenic translation programs. Taken together, these insightful findings bring us significantly closer to our goal of understanding how signaling pathways impact major tumors suppressors in the control of cell growth.

15. SUBJECT TERMS

Cell growth, breast cancer cells, p68DDX5, ribosomes

Table of Contents

INTRODUCTION

Cancers result from an inability of a cell to control its own growth. Normally, a cell interprets external and internal signals to create a balanced growth schedule. The main interpreters of these signals within a cell are called ARF and p53, and it falls on the shoulders of these two proteins to maintain normal cell growth (1, 2). In this sense, both ARF and p53 are tumor suppressors that constantly monitor the growth state of the cell. In mouse and human cancers, loss of the ARF tumor suppressor is second only to mutation of p53, providing critical evidence of ARF's role in both monitoring and preventing the outbreak of cancer cells. A common target of ARF is the NPM/B23 oncogene, an abundant protein of the nucleolus (3, 4). NPM normally responds to growth factors and, due to its nucleolar localization, is thought to transmit these growth signals to the maturing ribosome machinery (5, 6). Cells lacking *Arf* exhibit tremendous gains in ribosome production and subsequent protein synthesis (7). Moreover, the entirety of this growth phenotype is dependent on NPM and p68DDX5 expression in the nucleolus, with loss of either capable of completely reversing the phenotype back to normal (8). This exciting new finding indicates that ARF is a master regulator of cell growth through its tight control of NPM- or DDX5-directed ribosome production and export. Importantly, we have found NPM overexpressed in nearly 50% of breast carcinomas that we have analyzed, implying that dysregulation of NPM may be a key event in promoting breast cancer development. In effect, tumor cells that require increased protein synthesis might accumulate more NPM or DDX5 in an attempt to increase ribosome output. It is our goal to determine whether NPM directly regulates ribosome maturation to promote breast cancer formation and to establish the importance of ARF in deterring this effect. We propose to now determine the complex roles of ARF, DDX5, and NPM in the nucleolus of breast epithelial cells and how they impact both ribosome biogenesis and cell growth to prevent and/or promote tumorigenesis.

This work has tremendous clinical implications as *Arf* (9p21) and *p68Ddx5* (17q24) reside on loci that are either deleted or amplified in ER+ resistant breast tumors, respectively. This fact makes our basic science on this interesting growth network directly applicable to the breast cancer phenotype/genotype.

BODY

As stated in the approved Statement of Work, we focused our energies on the tasks planned for Months 1-48. These included experiments outlined in Tasks 1, 3 and 4. In this fourth Annual Progress Report, we detail the progress and results from these studies.

Task 1. Determine the role of ARF in suppressing breast tumor formation (Months 1-30):

e. Determine the influence of ARF on Drosha-mediated RNA translation (Months 1-16).

During the fourth year of this grant, we have focused our efforts more broadly on completing the experiments outlined in Task 1. ARF is a multifunctional tumor suppressor that acts as both a sensor of oncogenic stimuli and as a key regulator of ribosome biogenesis. Recently, our group established the DEAD-box RNA helicase and microRNA (miRNA) microprocessor accessory subunit, DDX5, as a critical target of basal ARF function. To identify other molecular targets of ARF, we focused on known interacting proteins of DDX5 in the microprocessor complex. Drosha, the catalytic core of the

microprocessor complex, plays a critical role in the maturation of specific non-coding RNAs, including miRNAs and rRNAs. Here, we report that chronic or acute loss of *Arf* enhanced Drosha protein expression. This induction did not involve Drosha mRNA transcription or protein stability but rather relied on the enhanced translation of existing Drosha mRNAs. Enhanced Drosha expression was required to maintain increased rRNA synthesis and cellular proliferation. *Arf*-deficient cells transformed by oncogenic RasV12 were dependent on increased Drosha expression as Drosha knockdown was sufficient to completely inhibit Ras-dependent cellular transformation. Thus, we propose that ARF controls Drosha translation to prevent aberrant cell proliferation and Ras-dependent transformation.

Previous reports have demonstrated that ARF serves as a major regulator of ribosome biogenesis (9) and that this is due at least in part to its modulation of DDX5 localization and function (8). Moreover, other studies have established a clear link between DDX5 and Drosha in the processing of double stranded RNAs including rRNAs (10, 11). To test whether a relationship exists between Drosha and ARF, we first compared Drosha protein levels using WT and *Arf* $^{-/-}$ MEFs. Drosha protein induction was observed upon the genetic ablation of the *Arf* locus (Figure 1a, left). We explored this phenomenon further by acutely manipulating the expression of ARF using either a lentivirus encoding shRNAs targeting *Arf* exon 1β, the ARF-specific exon of the *CDKN2A* locus, or an ARF overexpressing retrovirus. Consistent with the previous finding, knockdown of basal ARF expression resulted in heightened Drosha expression (Figure 1b). Conversely, ectopic overexpression of ARF lowered Drosha expression (Figure 1c). ARF-mediated regulation of Drosha protein expression was not MEF-specific as similar trends were observed using *Arf* $^{flox/flox}$ mouse astrocytes infected with adenoviruses encoding Cre recombinase (Figure 1d).

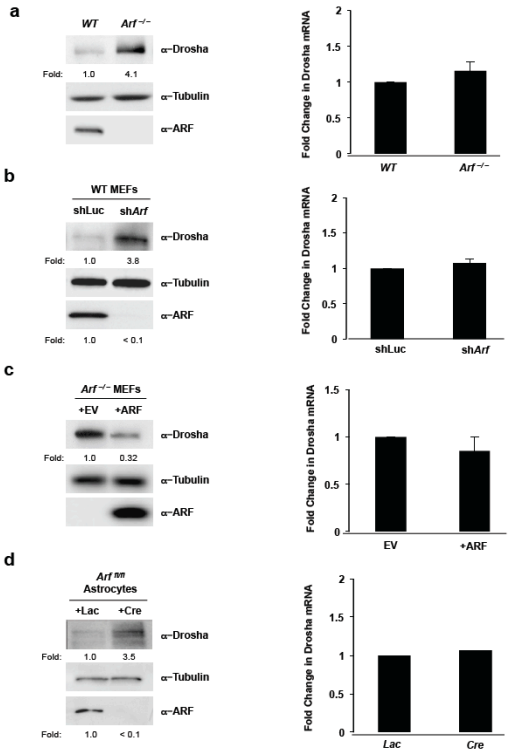

Figure 1. *Arf* negatively regulates Drosha protein expression in a transcriptionally independent manner. (a-d, left column) Genetic ablation of the Arf locus, ARF knockdown, ARF ectopic expression, and cre-mediated excision of the Arf gene collectively identified an inverse relationship between Arf and Drosha protein levels. Cells were lysed, and separated proteins were immunoblotted for the indicated proteins. Drosha expression fold change relative to WT / control infected cells is indicated. (a-d, right column) Drosha mRNA levels are essentially unchanged under all tested conditions despite the observed differences in Drosha protein levels suggesting that ARF posttranscriptionally regulates Drosha. First-strand cDNA was synthesized from isolated total RNA, and quantitative RT-PCR analysis was performed. Drosha mRNA levels were normalized to *Gadph* mRNA levels. Fold change was calculated using the $\Delta\Delta C_T$ method. Data are the mean ± standard deviation of three independent experiments.

We compared the percentage of Drosha mRNA transcripts associated with actively translating polyribosomes (polysomes) in WT and *Arf* $^{-/-}$ MEFs. Ribosomes were detected in lysates separated in sucrose gradients by continuous measurement of RNA absorbance. Loss of *Arf* enhanced the overall formation of polysomes actively engaged in mRNA translation as previously described (Figure 2b). Quantitative RT-PCR was performed to evaluate the distribution of Drosha mRNA transcripts in monosome-, disome-, and polysome-containing fractions. *Drosha* mRNA was abundant in the heavier polysome fractions in the absence of *Arf*, shifting from lighter polysomes in fraction 9 from WT cells to heavy fractions 11-13 in *Arf* $^{-/-}$ cells (Figure 2c). Importantly, GAPDH mRNA transcript distribution remained unchanged across polysomes (Figure 2d), suggesting that *Arf*-loss does not globally affect the translation of every cellular transcript, but rather leads to selective mRNA translation.

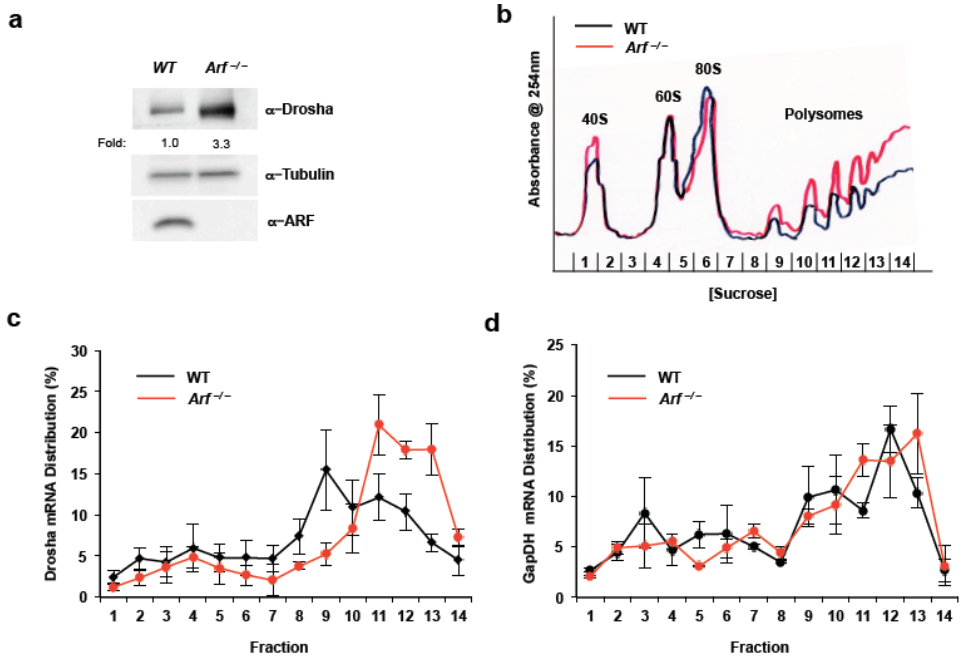

Figure 2. Translation of Drosha mRNAs is augmented upon loss of *Arf*. (a) Endogenous Drosha protein levels are elevated in MEFs that lack *Arf* compared to WT MEFs. (b) In the absence of *Arf*, there is an overall increase in mRNA-association with actively translating polysomes. Cytosolic extracts were prepared from equal number WT and *Arf* $^{-/-}$ MEFs that had been treated for 5 min with cycloheximide (10 μg/ml) Extracts were then subjected to differential density centrifugation and analyzed via constant UV monitoring (254 nm). (c-d) Drosha mRNA is more concentrated in the heavier sucrose fractions (11-13) in *Arf* $^{-/-}$ MEFs indicating an increase in Drosha translation under these conditions. Importantly, GAPDH translation remained unchanged . Total RNA was isolated from each sucrose gradient fraction, and first-strand cDNA was synthesized for each fraction. Monosome-, disome-, and polysome-associated Drosha mRNA levels were measured with qRT-PCR and were calculated as a percentage of total Drosha mRNA present in all fractions. Data are the mean ± SEM of three separate experiments.

ARF protects normal cells from oncogenic RasV12 transformation by activating a classic p53-dependent growth arrest or apoptotic response (12). However, in the absence of *Arf*, MEFs transduced with RasV12 undergo cellular transformation, an event that can be both observed and quantified by anchorage independent growth in soft agar (13). To determine whether elevated Drosha levels phenocopied *Arf* loss, WT MEFs were infected with a retroviruses encoding Drosha in combination with

oncogenic RasV12 and then plated in soft agar. Unlike RasV12-transduced *Arf* $^{-/-}$ MEFs, Drosha and RasV12-infected WT cells failed to form colonies. Furthermore, unlike RasV12, Drosha was unable to stimulate transformation of *Arf* $^{-/-}$ MEFs suggesting that Drosha does not act as a bona fide oncogene (Figures 3a & 3b).

While elevated levels of Drosha were not sufficient to transform cells, we hypothesized that Drosha might be necessary for transformation in the absence of *Arf*. To test this hypothesis, *Arf* $^{-/-}$ MEFs were first infected with retroviruses encoding oncogenic RasV12 followed by infection with lentiviruses encoding Drosha-specific shRNAs (Figure 3c). Reduction of Drosha protein expression was sufficient to impair RasV12-driven colony formation and anchorage-independent growth as indicated by a reduction in both the number of colonies and their overall size (Figure 3d & 3e), implying that *Arf*-deficient cells transformed by oncogenic RasV12 require elevated Drosha expression to maintain the transformed phenotype.

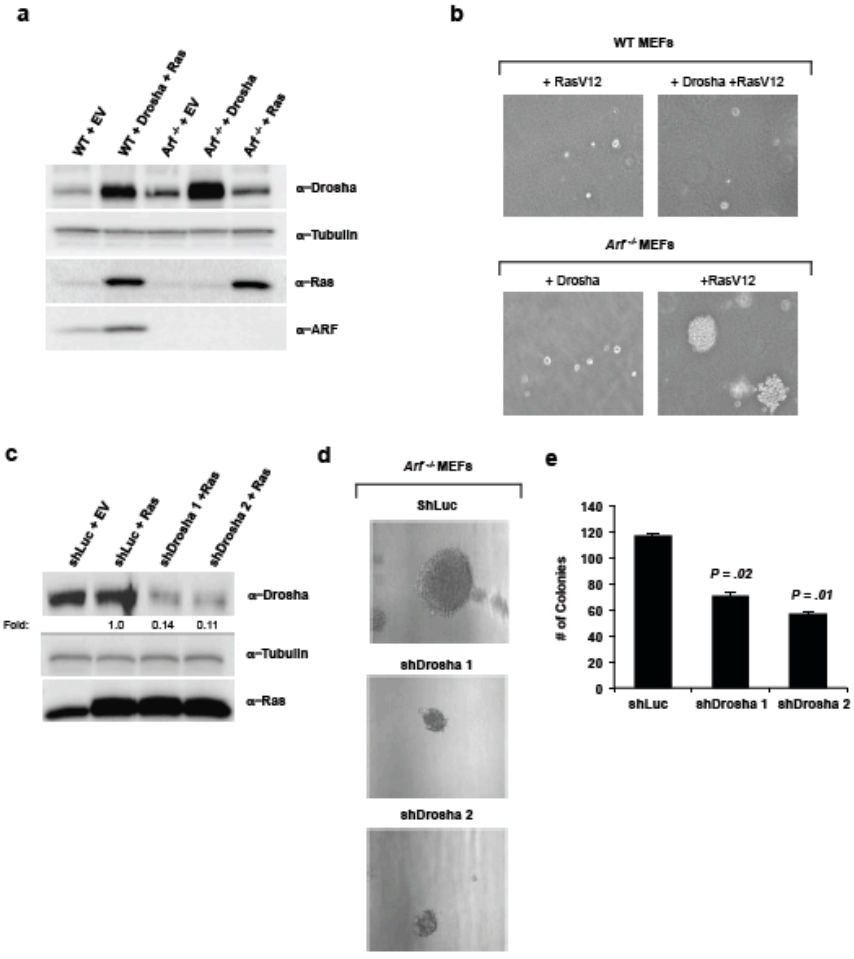

Figure 3. Drosha knockdown significantly inhibits Ras-induced colony formation of *Arf* $^{-/-}$ MEFs. (a-b) WT and *Arf* $^{-/-}$ MEFs transduced with Drosha and Ras or only Drosha, respectively, failed to form colonies in soft agar (c) *Arf* $^{-/-}$ MEFs were infected with RasV12, then subsequently infected with shDrosha or control shLuc. Ras overexpression and Drosha knockdown were confirmed by Western blot. (d-e) A reduction in Drosha expression significantly impairs both the colony formation and the colony size of *Arf* $^{-/-}$ MEFs that have been transformed with RasV12. For these experiments, a total of 50^4 Infected cells per condition were seeded in triplicate onto soft agar plates and were grown for 3 weeks. Colonies were examined under a microscope and counted. Number of colonies is expressed as the mean ± standard error of the mean.

(14)

f. Identify the signalling pathway(s) responsible for enhanced ARF mRNA translation

(Months 11-16).

The ARF tumor suppressor is a potent sensor of hyper-proliferative cues emanating from oncogenic signaling (14). ARF responds to these cues by eliciting a cell cycle arrest, effectively abating the tumorigenic potential of these stimuli. We now show that ARF protein is still induced in response to Ras^{V12} in the absence of *Dmp1* through the enhanced translation of existing *Arf* mRNAs. Hyper-activation of mTORC1 through *Tsc1* loss resulted in a significant increase in ARF expression, activation of the p53 pathway, and a dramatic cell cycle arrest, which were completely reversed upon *Arf* deletion. ARF protein induced from Ras^{V12} in the absence of *Dmp1* repressed anchorage independent colony formation in soft agar and tumor burden in an allograft model. Taken together, our data demonstrate the ability of the ARF tumor suppressor to respond to hyper-growth stimuli to prevent unwarranted tumor formation.

Our data indicate that ARF is induced in response to oncogenic Ras^{V12} independently of Dmp1 transcriptional activity. We hypothesized that the phosphatidylinositol-3-kinase/mammalian target of rapamycin (PI3K/mTOR) signal transduction pathway could potentially regulate ARF expression. mTOR is the critical cell growth regulatory pathway that coordinates ribosome biogenesis and mRNA translation. Regulation by this pathway is often associated with translational control of target genes whose protein levels, but not mRNA levels, are modulated in particular cellular contexts (15). To begin evaluating this pathway, wild-type MEFs were transduced with Ras^{V12} and then treated with LY294002, a pharmacological inhibitor of PI3K, for 24 hours prior to harvesting. Decreased levels of phospho-S6 (Ser 240/244) demonstrated that downstream mTOR signaling was abrogated following LY294002 treatment (Fig 4A). Ras^{V12} robustly induced ARF protein levels, and this induction was abrogated with LY294002 treatment (Fig. 4A). Furthermore, wild-type and $Dmp1^{-/-}$ MEFs were transduced with Ras^{V12} and subsequently treated with rapamycin, the pharmacological inhibitor of mTOR, for 24 hours prior to harvesting. Repressed levels of phospho-S6 (Ser 240/244) revealed that mTOR signaling was disrupted from rapamycin exposure (Fig. 4B). In both genotypes, the induced levels of ARF protein expression were sensitive to rapamycin treatment (Fig. 4B), suggesting that mTOR signaling is essential for ARF's induction from Ras.

TSC1 forms a complex with TSC2 that negatively regulates mTOR signal transduction (16). We hypothesized that activation of the mTOR pathway by acute knockdown of TSC1 would induce ARF protein levels. To test this, wild-type MEFs were infected with lentiviruses encoding siRNAs recognizing *Tsc1*. Two hairpins were used to reduce TSC1 expression (Fig. 4C). ARF protein levels were up-regulated from transient knockdown of TSC1 in a dose dependent manner (Fig. 4C). Also, $Tsc1^{flox/flox}$ MEFs were infected with adenoviruses encoding Cre recombinase or a β-galactosidase (LacZ) control. Enhanced levels of phospho-S6 (Ser 240/244) demonstrated that hyper-activation of mTOR signaling occurred from loss of *Tsc1* (Fig. 4D). Genetic ablation of *Tsc1* also caused an increase in ARF protein levels (Fig. 4D), corroborating the results observed from using RNAi against *Tsc1.* Moreover, we infected wild-type MEFs with LacZ or Cre to ensure that this finding was not a nonspecific off-target effect of Cre recombinase or adenoviral infection protocol (Fig. 4E). Additionally, $Tsc1^{+/flox}$ and $Tsc1^{flox/flox}$ were infected with Cre or LacZ to evaluate a dose dependent loss of *Tsc1* on ARF protein levels (Fig. 4F). Loss

of one copy of *Tsc1* was sufficient to induce ARF protein expression, while loss of both copies of *Tsc1* induced ARF protein expression to a greater extent (Fig. 4F). To investigate whether the ARF induction observed from the loss of *Tsc1* is dependent on TSC/mTOR signaling, we infected *Tsc1*$^{flox/flox}$ MEFs with Cre or LacZ control and then treated with rapamycin for 24 hours prior to harvesting. Diminished levels of phospho-S6 (Ser 240/244) demonstrated that rapamycin successfully blocked mTOR signaling (Fig. 4G). As seen before with Ras^{V12} infection (Fig. 4B), ARF protein levels induced from the loss of *Tsc1* were sensitive to rapamycin treatment (Fig. 4G).

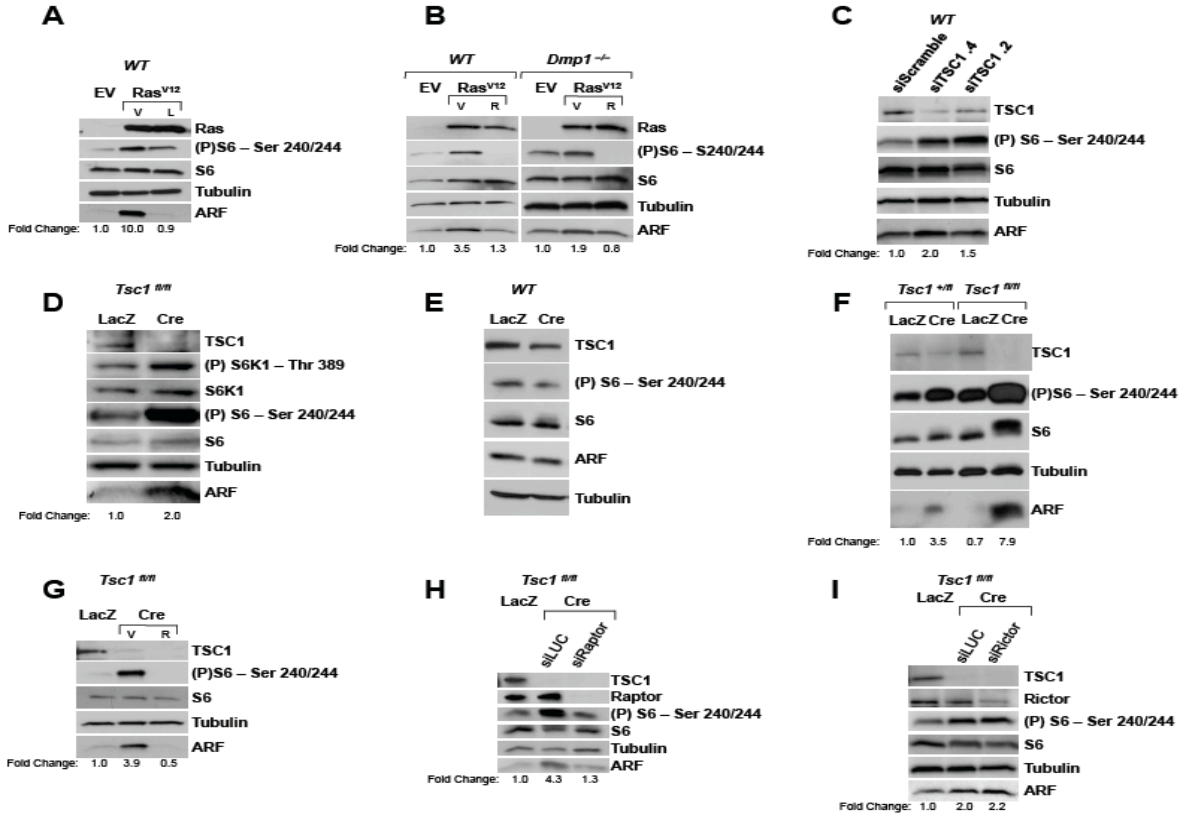

Figure 4: **Ras/PI3K/TSC/mTORC1 pathway can regulate ARF.** (A) Wild type (WT) MEFs were infected with retroviruses encoding empty vector or Ras^{V12} and were harvested. Cells were treated with LY294002 or vehicle for 24 hours prior to harvesting. (B) WT or *Dmp1*$^{-/-}$ MEFs were infected with retroviruses encoding empty vector or Ras^{V12} and were harvested at five days post infection. Ras^{V12} infected cells were treated with rapamycin or vehicle for 24 hours prior to harvesting. (C) Wild type MEFs were infected with lentiviruses encoding short hairpins against *Tsc1* or siScrambled control and were harvested at seven days post infection. (D-E) *Tsc1*$^{flox/flox}$ or WT MEFs were infected with adenoviruses encoding β-galactosidase (LacZ) or Cre recombinase and were harvested at nine days post infection. (F) *Tsc1*$^{+/flox}$ or *Tsc1*$^{flox/flox}$ MEFs were infected with adenoviruses encoding β-galactosidase (LacZ) or Cre recombinase and were harvested at nine days post infection. (G) *Tsc1*$^{flox/flox}$ MEFs were infected with adenoviruses encoding β-galactosidase (LacZ) or Cre recombinase and harvested at nine days post infection. Cre infected cells were treated with rapamycin or vehicle control for 24 hours prior to harvesting. (H-I) *Tsc1*$^{flox/flox}$ MEFs were infected with adenoviruses encoding β-galactosidase (LacZ) or Cre recombinase. Cre infected cells were then transduced with short hairpins recognizing *Raptor* (H) or *Rictor* (I) or siLUC control, and then harvested for western blot analysis.

mTOR assembles into two different protein complexes, mTORC1 and mTORC2, each of which are

reported to serve unique functions in the cell (17). mTORC1 contains Raptor, LST8, Deptor, PRAS40, and mTOR, and is critical for regulating protein synthesis; mTORC2 includes Rictor, LST8, Deptor, Protor, Sin1, and mTOR, and plays a role in cytoskeletal organization. RNA interference was used to acutely knockdown *Raptor* or *Rictor* in order to respectively assess the contributions of mTORC1 or mTORC2 following *Tsc1* deletion (Fig. 4H and Fig. 4I). Acute knockdown of *Raptor*, but not *Rictor*, abrogated the induction of ARF expression from the ablation of *Tsc1* (Fig. 4H and Fig. 4I). This suggests that mTORC1, but not mTORC2, is necessary for mediating mTOR induction of ARF. Taken together, these data suggest that hyper-activation of Ras/TSC/mTORC1 pathway can regulate ARF protein levels.

To further test the hypothesis that translational regulation could be the molecular mechanism responsible for eliciting ARF's induction from mTOR hyperactivation, we assessed the association of *Arf* mRNA with actively translating polyribosomes. To accomplish this task, cytosolic ribosomes were isolated by sucrose gradient centrifugation from equal numbers of *Dmp1*$^{-/-}$ MEFs infected with either Ras^{V12} or an empty vector control. Ribosomal RNAs were detected by continuous UV monitoring of cytosolic rRNAs' absorbance [A_{254nm}] (Fig 5A). To assess the distribution of *Arf* mRNA transcripts in individual fractions comprising isolated monosomes, disomes, or polysomes, total RNA was isolated from each sucrose gradient fraction and *Arf* mRNA levels were determined with qRT-PCR. Strikingly, *Arf* mRNA transcripts associated with different polyribosome fractions in Ras^{V12}-infected and empty vector-infected *Dmp1*-null cells (Fig 5B). In Ras^{V12}-infected *Dmp1*$^{-/-}$ MEFs, *Arf* mRNA was pooled to a heavier polyribosome fraction, indicating that there is a greater extent of *Arf* mRNAs being actively translated by multiple ribosomes (more ribosomes associated per mRNA) in these cells (Fig. 5B). These data support the hypothesis that ARF is translationally regulated in the presence of oncogenic Ras^{V12} signals.

To address the possibility that general gains in global protein translation could account for the increased translation of *Arf* mRNA transcripts, we evaluated the distribution of *Gapdh* mRNA in sucrose gradient fractions in *Dmp1*$^{-/-}$ MEFs infected with either Ras^{V12} or an empty vector control (Fig 5C). No dramatic differences in the distribution of *Gapdh* mRNA transcripts were observed across isolated monosomes or polyribosomes, in contrast to the distribution observed for *Arf* mRNA (Fig 5C). This suggests that the gain in *Arf* mRNA association with actively translating polyribosomes is a selective phenotype caused by Ras^{V12} oncogenic signaling in the absence of *Dmp1*.

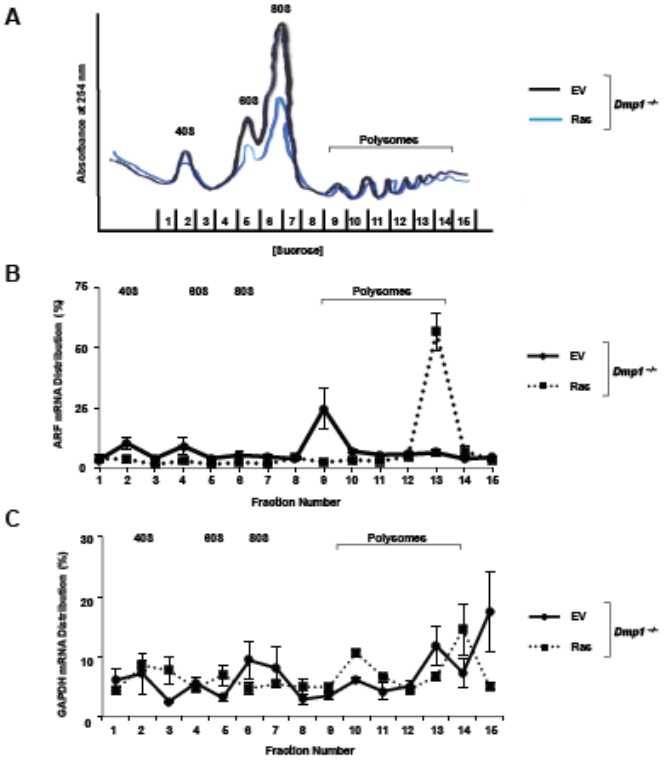

Figure 5: ARF mRNA association with actively translating polyribosomes increases with hyper-growth stimuli. *Dmp1*$^{-/-}$ MEFs were transduced with retroviruses encoding empty vector or Ras^{V12} and were harvested at five days post infection. Cytosolic extracts from equal number of cells ($3x10^6$) treated for 5 minutes with cycloheximide (10 µg/mL) were separated on 7 – 47% sucrose gradients with constant UV monitoring. (A) The representative graph depicts the A_{254} absorbance of ribosome subunits over increasing sucrose density. (B) Total RNA was isolated from each sucrose gradient fraction. Monosome, disome, and polysome associated *Arf* mRNA were measured with qRT-PCR and were calculated as percentage of total *Arf* mRNA collected in all fractions. Data are the mean +/– S.E.M. of three independent experiments, and *P* values are calculated using Student *t*-test. (C) Monosome, disome, and polysome associated *Gapdh* mRNA were measured as in (B).

Task 3. Establish the oncogenic potential of the p68DDX5 RNA helicase (Months 24-48).

c. Examine the role of p68 in breast cancer cell growth (Months 36-48).

We previously identified an interaction between NPM and DDX5 while probing for NPM binding partners (5). Like DDX5, NPM is a multifunctional protein with key roles at multiple stages of ribosome biogenesis. NPM associates with the rDNA locus (18), regulating transcription and processing of the rRNA (3). Further, NPM functions as a nuclear export chaperone for ribosomes (5), a function that is antagonized by ARF (4). Interestingly, early embryonic lethality is a phenotype of both *Npm1*-/- and *Ddx5*-/- mice (11, 19, 20). We hypothesized that ARF impaired DDX5 function through regulation of its interaction with NPM.

Given the ability of ARF to regulate both proteins individually, we tested whether ARF effected the interaction between DDX5 and NPM. Comparison of *WT* and *Arf*-/- MEF lysates by co-immunoprecipitation revealed that ARF significantly reduced the interaction of DDX5 with NPM (Fig. 6A). We then sought to determine the NPM-binding domain on DDX5 to assess whether this interaction was critical for the growth-stimulatory abilities of DDX5. Little has been reported on the proteins that interact with DDX5 through its C-terminal domain. Given the possibility that core domain mutations might directly impair conserved features that are critical in the DEAD-box helicase family and complicate any interpretations of its overall importance, we instead focused on mutations in the C-terminus. A panel of overlapping C-terminal deletion mutations was introduced to DDX5 in a GST-fusion protein

expression vector. *In vitro* immunoprecipitation reactions using His-tagged NPM and GST-DDX5 or its mutants mapped an NPM interaction motif to residues 500-610 at the C-terminus of DDX5 (Fig. 6B). For further experiments, we chose a smaller mutant within this domain, DDX5Δ520-550. While ectopically expressed Flag-DDX5 interacted with endogenous NPM in *Arf*-/- MEFs, the Δ520-550 mutant displayed no visible interaction (Fig. 6C). Flag-DDX5-Δ520-550 also had reduced occupancy of the rDNA promoter compared to wild-type Flag-DDX5 (Fig. 6D), and did not stimulate 47S pre-rRNA transcription (Fig. 6E). Further, while Flag-DDX5 associated with nuclear pre-ribosomal fractions containing the 40S and 60S ribosomal subunits, Flag-DDX5-Δ520-550 was almost completely absent from the 60S fractions containing the large ribosomal protein rpL7a (Fig. 6F). Finally, in transduced *Arf*-/- MEFs, Flag-DDX5-Δ520-550 expression did not affect proliferation compared to the empty vector control, whereas Flag-DDX5 expression enhanced proliferation (Fig. 6G). Thus, it appears that DDX5 cooperates with NPM, through a direct interaction that is antagonized by ARF, to stimulate rRNA synthesis and proliferation.

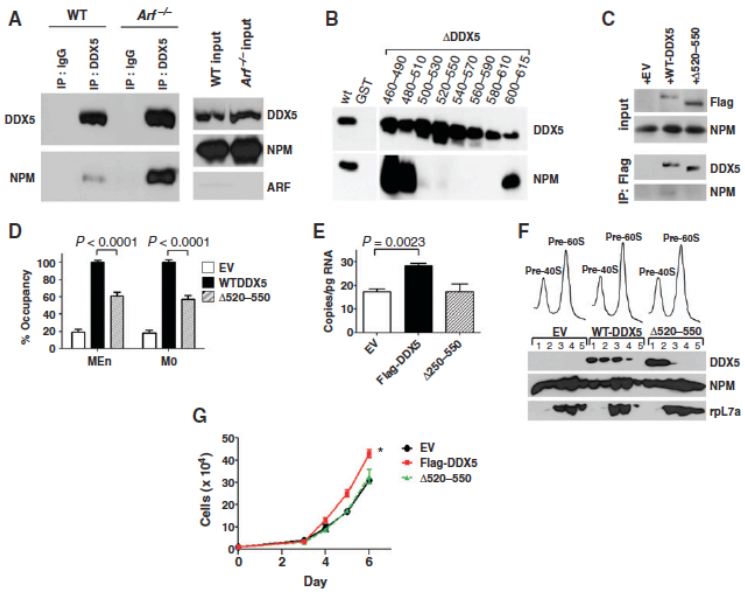

Figure 6: The ARF-regulated interaction between DDX5 and NPM is required for the growth-stimulatory effects of DDX5. **A,** The interaction between DDX5 and NPM was compared in lysates from WT and *Arf*-/- MEFs by co-immunoprecipitation with antibodies against DDX5. **B,** A panel of GST-Flag-DDX5 mutants were subjected to GST-pull-down following incubation with His-NPM proteins and immunoblotting was performed with antibodies recognizing DDX5 and NPM. **C,** *Arf*-/- MEFs expressing Flag-DDX5 or Flag-DDX5-Δ520-550 were subjected to immunoprecipitation with an antibody against the Flag epitope. **D,** *Arf*-/- MEFs were subjected to chromatin immunoprecipitation reactions with an antibody against the Flag epitope. DNA recovered from the reactions was subjected to QPCR using primers to two different areas, MEn and M0, within the rDNA promoter. **E,** QPCR was performed for the 47S pre-rRNA from total RNA isolated from wild-type MEFs. **F,** Nuclei from *Arf*-/- MEFs expressing Flag-DDX5 or Flag-DDX5-Δ520-550 were subjected to sucrose gradient centrifugation. Expression of rpL7a or Flag-tagged proteins in the isolated fractions was determined by western blot. **G,** *Arf*-/- MEFs were plated in triplicate at 20,000 cells per well for a proliferation assay and counted daily over a time course. *, *P*=0.0058.

d. Develop a high-throughput assay for p68 helicase activity (Months 36-48).

We have now completed preliminary studies with a newly generated luminescent readout for NPM-DDX5 interactions in cells. Using a split luciferase system, we are now able to screen chemical compounds or shRNA libraries for inhibitors of the NPM-DDX5 interaction (Fig. 7).

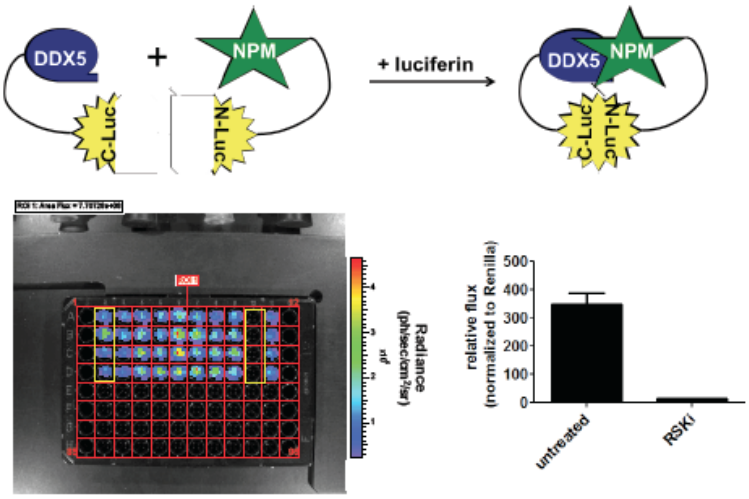

Figure 7: Characterization of the DDX5-NPM interaction by split-luciferase complementation assay. The ARF-regulated interaction with NPM is necessary for DDX5 to stimulate rRNA synthesis and proliferation (data not shown). We reasoned that given the diverse biological functions of DDX5, targeting its interaction with NPM may allow for inhibition of its growth-stimulatory involvement in ribosome biogenesis without compromising all of its attributed functions. To assess the DDX5-NPM interaction in living cells, we adopted the split luciferase complementation system. Transduction of DDX5- and NPM-fusion proteins linked to complementary N- and C-terminal luciferase polypeptides (N-Luc and C-Luc, respectively) recapitulates the enzymatic activity of luciferase such that upon addition of the substrate D-luciferin the cells emit radiance. Using this assay, we conducted a preliminary screen for compounds that disrupted DDX5-NPM-interaction-dependent photon emission. 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione hydrochloride, an inhibitor of ribosomal S6 kinase activation, reduced the DDX5-NPM interaction without affecting expression of the fusion proteins (data not shown). This proof-of-principle experiment illustrates the possibility of using the split luciferase complementation assay to identify pharmacological inhibitors of the DDX5-NPM interaction.

Task 4. Determine the contribution of ARF translational targets to breast cancer formation (Months 30-60).

a. Identify ARF translation targets (Months 30-42).

We have recently identified a novel translational target of the ARF tumor suppressor: the DHX33 DEAD-box RNA helicase. DHX33 is a pivotal DEAD-box RNA helicase in the multi-step process of RNA polymerase I-directed transcription of the ribosomal DNA locus. Changes in DHX33 expression directly influence the RNA polymerase I transcriptional machinery to regulate rRNA availability and ribosome biogenesis. As a central player in cellular growth, ribosome biogenesis is sensitive to oncogenic signals, such as those emanating from the RasV12 oncogene, and is constantly monitored by the ARF/p53 tumor suppressor pathway. In this report, we explored the coordinated regulation of DHX33 expression by RasV12 and ARF to determine DHX33's role in sensing these opposing signals to regulate ribosome biogenesis. In wild-type primary fibroblasts, RasV12 infection induced a transient increase in DHX33 protein level as well as rRNA transcriptional rate that was eventually suppressed by a delayed activation of the ARF/p53 pathway (Fig. 8).

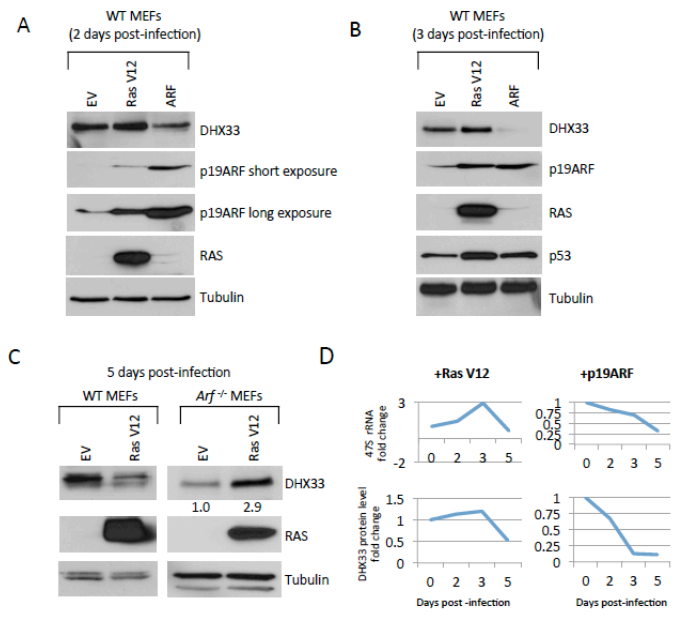

Figure 8: Regulation of DHX33 during oncogenic stress. **(A)** Wild type MEFs were infected by retrovirus encoding RasV12, $p19^{ARF}$, or empty vector, whole cell lysates were prepared and were subjected to western blot analysis with the indicated antibodies. **(B)** Above-mentioned cells were harvested 3 days post-infection and whole cell extracts were subjected to western blot analysis with the indicated antibodies. **(C)** Wild type MEFs or ARF null MEFs were infected by retrovirus encoding Ras V12 or empty vector, whole cell extracts were prepared and were subjected to western blot analysis with the indicated antibodies. **(D)** For wild type MEFs that have been infected by the above-mentioned retrovirus, total RNA was extracted at either 2 days, 3 days or 5 days post-infection. Mouse 47S pre-rRNA levels were analyzed by real time PCR and graphed in a time-dependent manner. Changes of DHX33 protein levels in the time course were also graphed after quantitation of DHX33 signals in Fig 8A-8C after normalization to the empty vector control.

DHX33 expression was exclusively controlled at the level of translation. ARF caused a dramatic reduction in polysome-associated DHX33 mRNAs while RasV12 led to a complete shift of existing DDX33 mRNAs to actively translating polysomes (Fig. 9).

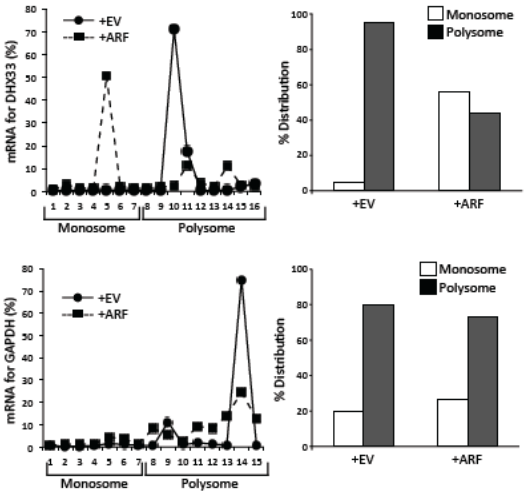

Figure 9. Translational control of DHX33 by ARF. Left, Fractions from either mono-ribosomes or polyribosomes isolated from cells infected with control (EV) or ARF retroviruses were subjected to RNA isolation and q-PCR analysis for DHX33 mRNA levels. GAPDH levels were used as a control. Data is presented as a percentage of mRNA from each fraction calculated from a standard curve generated by a series of diluted DHX33 plasmid. Right, Bar graph of total percentage of mRNA levels for DHX33 and GAPDH in either monoribosome or polysome fractions under each condition.

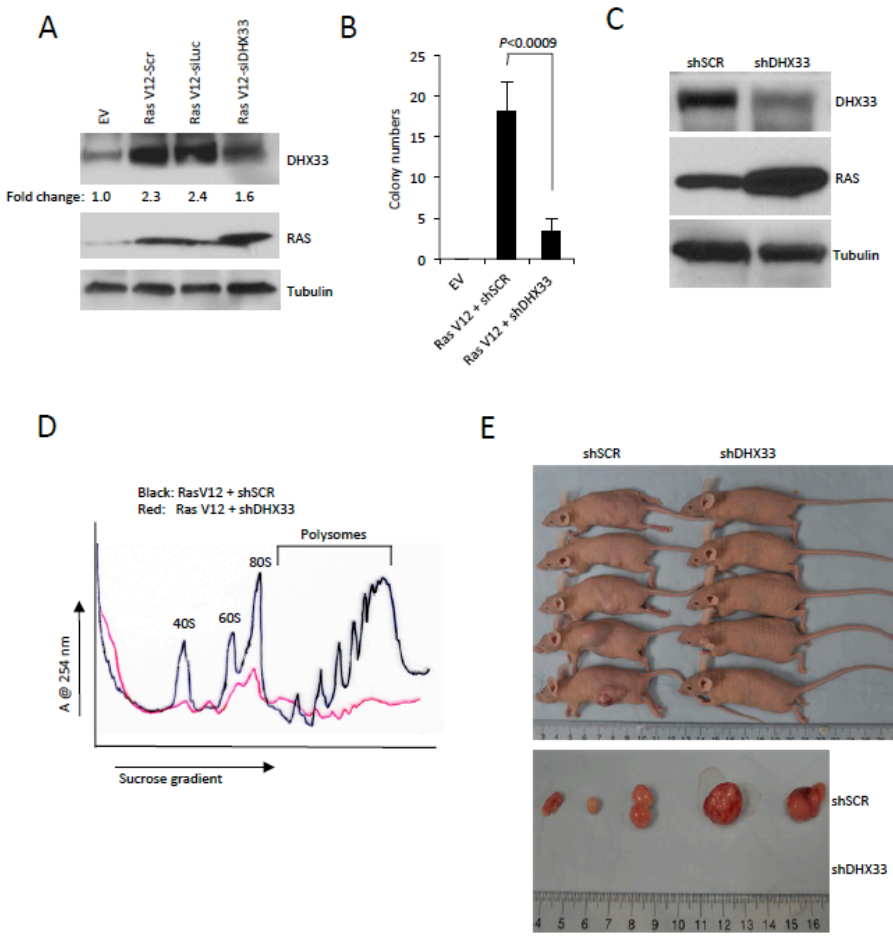

Figure 10. DHX33 upregulation is important for Ras-initiated tumor formation. (A) ARF null ear fibroblasts from 2-month old mouse were firstly infected by retrovirus encoding either pBABE-empty vector or pBABE-RasV12, cells were then infected by lentivirus encoding either shScrambled, shLuciferase or shDHX33. Whole cell lysates were extracted and analyzed by western blot with ant-Ras, anti-DHX33 and anti-tubulin. **(B)** Approximately 5000 above-mentioned cells were plated onto soft agar plates in triplicate in 60 mm dishes for detecting anchorage-independent cell growth. Quantitation of the colony numbers from 3 representative fields under 4X magnification, bars represent standard deviation calculated from 3 different numbers of colonies on triplicate plates. **(C)** NIH3T3 cells were firstly infected by Ras V^{12} retrovirus followed by infection with lentivirus encoding shScrambled or shDHX33. Whole cell lysates were subjected to western blot analysis with anti-Ras and anti-DHX33 antibodies with tubulin as a loading control. **(D)** Approximately 3 million NIH3T3 cells of the indicated infection were utilized for cytosolic polysome profiles. **(E)** Upper: One million Ras-infected NIH 3T3 cells after DHX33 knockdown were injected into one side of the flanks of NUDE mice with shScrambled as a control. Tumor formation were visualized and photographed after growing for 14 days. Lower: Mice were sacrificed at day 14 post-injection and tumor were excised out and photographed. Note: the first three mice had leakage after injection to various degrees.

Additionally, DHX33 knockdown abolished RasV12-induced rRNA transcription and protein translation and prevented both the in vitro and in vivo transforming properties of oncogenic RasV12 (Fig. 10). Our results directly implicate DHX33 as a crucial player in establishing rRNA synthesis rates in the face of RasV12 or ARF signals, adjusting ribosome biogenesis to match the appropriate growth or anti-growth signals.

KEY RESEARCH ACCOMPLISHMENTS

- ARF negatively regulates Drosha protein expression via a translational mechanism (Task 1e)
- Drosha knockdown significantly inhibits Ras-induced transformation in the absence of ARF (Task 1e)
- mTORC1 regulates the translation of ARF (Task 1f)
- The ARF-regulated interaction between DDX5 and NPM is required for the growth-stimulatory effects of DDX5 (Task 3c)
- We have characterized the DDX5-NPM interaction by a split luciferase complementation assay (Task 3d)
- We have identified DHX33 as a translational target of ARF (Task 4a)
- DHX33 upregulation in the absence of ARF is important for Ras-initiated tumor formation

REPORTABLE OUTCOMES

Manuscripts:

Saporita A.J., Chang H-C., Winkeler C.L., Apicelli A.J., Kladney R., Wang J.C., Townsend R.R., Michel L.S., and **Weber J.D.** (2011). RNA helicase DDX5 is a p53-independent target of ARF that participates in ribosome biogenesis. *Cancer Research,* 71: 6708-17.

Zhang Y., Forys J.T., Miceli A.P., Gwinn A.S. and **Weber J.D.** (2011). Identification of DHX33 as a mediator of rRNA synthesis and cell growth. *Molecular and Cellular Biology*, 31: 4676-91.

Miceli A.P., Saporita A.J., and **Weber J.D.** (2012). Hyper-growth mTORC1 signals translationally activate the ARF tumor suppressor checkpoint. *Molecular and Cellular Biology*, 32: 348-64.

Abstracts/Presentations: None

Patents/Licenses: None

Animal Models: In the fourth year, we have generated *Arf* $^{fl/fl}$*-Blg-Cre*, which will be free to any research that requests them.

Cell Lines: We have developed a unique primary mouse mammary stem cell line lacking the ARF tumor suppressor. These were established directly from *Arf* knockout mice on a both a pure C57Bl6 background and mixed C57Bl6/129 background. The *Arf*-null stem cells maintain a diploid phenotype and wild-type p53. These cells are spontaneously immortal and contain no artificial genes or plasmid constructs.

Funding Applied for: None

Employment Opportunities: None.

CONCLUSION

Our results provide a new perspective for understanding the tumor suppressor function of ARF, which has classically been thought of as a checkpoint sensor of hyperproliferative signals. The data presented here suggest that an equally important mechanism by which ARF functions as a tumor suppressor is to limit ribosome output as a defense against oncogene activation and the attendant enhanced cellular protein requirements. Whereas loss of *Arf* results in a cellular environment permissive toward oncogenic transformation, knockdown of Drosha or DHX33 can reduce susceptibility to transformation. Therefore, in the absence of *Arf*, Drosha and DHX33 become requisite non-oncogene effectors that promote an increased translational output in accord with the higher demand for protein production required upon oncogene activation. The ability of ectopic Drosha and DHX33 expression to stimulate ribosome biogenesis and growth in wild-type MEFs further proves the central role of Drosha and DHX33 in regulating this translational output.

Our data showing the growth-stimulatory functions of Drosha in ribosome biogenesis provides a strong rationale to explain the link between Drosha and cancer. We are just beginning a new analysis of breast tumors to evaluate any gains in DHX33 expression. Although still in its infancy, most non-oncogenes are thought of as critical regulators of cellular stress responses and that their expression provides cancer cells the means to tolerate multiple stresses (21). Drosha and DHX33 may represent a class of non-oncogenes whose activities are unleashed in the absence of crucial tumor suppressors. In this setting, the role of the Drosha non-oncogene is to make a required cellular process, such as ribosome biogenesis, more efficient or prolific in preparation for the tremendous protein synthesis demands following malignant transformation. It remains to be determined whether Drosha will be an efficacious target in the treatment of cancer; however our results validate its importance in supplying the sustained ribosome output required for oncogenic transformation. Finally, DDX5 participation in ribosome

biogenesis is negatively regulated by ARF, which inhibits the DDX5-NPM interaction, suggesting a dynamic interplay through which ARF and DDX5 duel for nucleolar growth control. We have established a unique split luciferase model system to begin to pre-clinically test compounds that might disrupt this interaction and prove efficacious in the treatment of cancers harboring ARF loss or DDX5 gain.

REFERENCES

1. Prives C & Hall PA (1999) The p53 pathway. *The Journal of pathology* 187(1):112-126.
2. Sherr CJ & Weber JD (2000) The ARF/p53 pathway. *Current opinion in genetics & development* 10(1):94-99.
3. Bertwistle D, Sugimoto M, & Sherr CJ (2004) Physical and functional interactions of the Arf tumor suppressor protein with nucleophosmin/B23. *Molecular and cellular biology* 24(3):985-996.
4. Brady SN, Yu Y, Maggi LB, Jr., & Weber JD (2004) ARF impedes NPM/B23 shuttling in an Mdm2-sensitive tumor suppressor pathway. *Molecular and cellular biology* 24(21):9327-9338.
5. Maggi LB, Jr*., et al.* (2008) Nucleophosmin serves as a rate-limiting nuclear export chaperone for the Mammalian ribosome. *Molecular and cellular biology* 28(23):7050-7065.
6. Yu Y*, et al.* (2006) Nucleophosmin is essential for ribosomal protein L5 nuclear export. *Molecular and cellular biology* 26(10):3798-3809.
7. Apicelli AJ*, et al.* (2008) A non-tumor suppressor role for basal p19ARF in maintaining nucleolar structure and function. *Molecular and cellular biology* 28(3):1068-1080.
8. Saporita AJ*, et al.* (2011) RNA helicase DDX5 is a p53-independent target of ARF that participates in ribosome biogenesis. *Cancer research* 71(21):6708-6717.
9. Sugimoto M, Kuo ML, Roussel MF, & Sherr CJ (2003) Nucleolar Arf tumor suppressor inhibits ribosomal RNA processing. *Molecular cell* 11(2):415-424.
10. Shiohama A, Sasaki T, Noda S, Minoshima S, & Shimizu N (2007) Nucleolar localization of DGCR8 and identification of eleven DGCR8-associated proteins. *Experimental cell research* 313(20):4196-4207.
11. Fukuda T*, et al.* (2007) DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. *Nature cell biology* 9(5):604-611.
12. Palmero I, Pantoja C, & Serrano M (1998) p19ARF links the tumour suppressor p53 to Ras. *Nature* 395(6698):125-126.
13. Kamijo T*, et al.* (1997) Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* 91(5):649-659.
14. Zindy F*, et al.* (1998) Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes & development* 12(15):2424-2433.
15. Spriggs KA, Bushell M, & Willis AE (2010) Translational regulation of gene expression during conditions of cell stress. *Mol Cell* 40(2):228-237.
16. Tee AR*, et al.* (2002) Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. *Proc Natl Acad Sci U S A* 99(21):13571-13576.
17. Ma XM & Blenis J (2009) Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* 10(5):307-318.

18. Murano K, Okuwaki M, Hisaoka M, & Nagata K (2008) Transcription regulation of the rRNA gene by a multifunctional nucleolar protein, B23/nucleophosmin, through its histone chaperone activity. *Mol Cell Biol* 28(10):3114-3126.
19. Grisendi S*, et al.* (2005) Role of nucleophosmin in embryonic development and tumorigenesis. *Nature* 437(7055):147-153.
20. Colombo E*, et al.* (2005) Nucleophosmin is required for DNA integrity and p19Arf protein stability. *Mol Cell Biol* 25(20):8874-8886.
21. Luo J, Solimini NL, & Elledge SJ (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* 136(5):823-837.

APPENDICES

None